TABLE OF CONTENT

1. INTRODUCTION 1
2. MATERIALS AND METHODS 4
2.1. Collection and identification of plant materials 4
2.2. Preparation of extracts 4
2.3. Qualitative phytochemicals analysis 4
2.4. Quantitative phytochemicals analysis 4
2.5. Preparation of saponin rich extract 4
2.6. Quantitative chromatographic analysis of saponin rich extracts 5
2.7. Residual solvents estimation in methanol and saponin rich extracts 5
2.8. Animal 5
2.9. Determination of maximum tolerable dose 5
2.10. Preliminary pharmacological evaluation 5
2.11. *In vitro* antiurolithiatic activity 5
2.12. *In vivo* antiurolithiatic activity 6
2.12.1. Ethylene glycol induced nephrolithiasis 6
2.12.2. Calcium oxalate seeds induced urolithiasis 7
2.12.3. Pharmacological activity of saponin rich fraction 9
2.13. Statistical analysis 11
3. RESULT AND DISCUSSION 12
3.1. Characteristics and qualitative phytochemical analysis of prepared extracts 12
3.2. Quantitative phytochemical analysis 12
3.3. Quantitative chromatographic analysis of saponin rich extracts 14
3.4. Residual solvents estimation in methanol and saponin rich extracts 14
3.5. Determination of maximum tolerable dose 15
3.6. Preliminary pharmacological evaluation 15
3.7. Evaluation of antiurolithiatic activity by *in vitro* model 15
3.7.1. Effects of methanolic extract on *in vitro* crystallization 16
3.7.2. Effect of saponin rich fraction of on *in vitro* crystallization 16
3.8. Evaluation of antiurolithiatic activity by *in vivo* model 17
3.8.1. Effect of methanolic extract on ethylene glycol induced urolithiasis 17
3.8.2. Effect of methanolic extract on calcium oxalate seeds induce urolithiasis 20
3.8.3. Effect of saponin rich fraction on ethylene glycol induced urolithiasis 23
3.8.4. Effect of saponin rich fraction CaOx seeds induced urolithiasis 26
3.9. Evaluation of pharmacological activities of saponin rich fraction 29
3.9.1. Evaluation of anti-inflammatory activity of saponin rich fraction 29
3.9.2. Evaluation of diuretic activity of saponin rich fraction 29
3.9.3. Evaluation of *in vitro* spasmolytic activity of saponin rich fraction 30
3.9.4. Evaluation of *in vitro* antioxidant activity 30
4. CUNCLUSION 32
5. REFERENCES 33

1. INTRODUCTION

Humankind is known to be afflicted by urinary stone disease, which was first noted in Egyptian mummies dated to 4800 BCE. As is clear from these historical clues, urinary stone disease has always been a common disease. Currently urinary stone formation affects 10% to 12% of the population in industrialized countries and the peak incidence seems to be at ages 20 to 40 years.[1] Calcium-containing stones, especially calcium oxalate monohydrate *(Whewellite)*, calcium oxalate dihydrate *(Weddellite)* are the most commonly occurring ones to an extent of 75-90% followed by magnesium ammonium phosphate *(Struvite)* to an extent of 10-15%, uric acid 3-10% and cystine 0.5-1%. In most of the cases the commonly occurring stones are calcium oxalate type.[2]

In India, approximately 5 -7 million patients suffer from stone disease and at least 1/1000 of Indian population needs hospitalization due to kidney stone disease. In India, the "stones belt" occupies parts of Maharashtra, Gujarat, Punjab, Haryana, Delhi and Rajasthan. In these regions, the disease is so prevalent that most of the members of a family will suffer from kidney stones sometime in their lives and with increasing number of stone cases in Gujarat population.[3]

The mechanisms involved in the formation of calcific stones are not fully understood but it is generally agreed that urinary lithiasis is a complex process involving events leading to crystal nucleation, aggregation and growth of insoluble particles.[4] Urine is always supersaturated with common stone forming minerals, however, the crystallization inhibiting capacity of urine does not allow urolithiasis to happen in most of the individuals, whereas, this natural inhibition is in deficit in stone formers.[5] Studies have also shown that tubular cell injury facilitates CaOx crystal formation and deposition in the renal tubules.[6] Animal and tissue culture studies have demonstrated that both oxalate and CaOx crystals directly induce renal epithelial cell injury mediated through lipid peroxidation and involve oxygen free radical generation.[7]

The most serious problem other than treatment for this disease is the high recurrence rate, which is more than 50% after 10 years.[8] Currently this serious problem of recurrence can be treated with extracorporeal procedures, such as extracorporeal SWL, endourological procedures such as ureteroscopy or percutaneous extraction procedures. SWL has really revolutionized urological practice in the last 20 years and open surgery for stone disease is considered unusual today.[9]

However, SWL might show some significant side effects, such as the traumatic effects of shock waves, severe hematuria, pancreatitis, infection and persistent residual

fragments that may serve as a nidus for new stone formation after SWL. In addition, large perforation of the collecting system, extravasation of irrigating fluid, urosepsis, ureteral injury and delayed bleeding can be summarized as the possible complications of endourological and percutaneous procedures.[1] To minimize these challenging side effects recurrent disease should be prevented.

Although some oral medications have positive effects but they are not effective in all patients. Oral citrate is one of the most widely used medical therapies for preventing urinary stone disease. It exerts its preventive effect through increasing urinary pH, decreasing Tamm-Horsfall protein aggregation and decreasing crystal adhesion to tubular cells.[10] However, this drug is not tolerated by all patients and some patients are still active stone formers during this therapy.[11]

In spite of substantial progress in the study of the biological and physical manifestations of kidney stones, there is still no satisfactory drug to use in clinical therapy. In addition, even though drug treatment has shown some feasibility in many randomized trials, it is not accomplished without side effects. Data from *in vitro*, *in vivo* and clinical trials reveal that phytotherapeutic agents could be useful as either an alternative or an adjunctive therapy in the management of urolithiasis.[12]

Medicinal plants have played a significant role in various ancient traditional systems of medication. Even today, plants provide a cheap source of drugs for majority of world's population. Several pharmacological investigations on the medicinal plants used in traditional antiurolithic therapy have revealed their therapeutic potential in the *in vitro* or *in vivo* models.[13] *Pedalium murex* Linn. and *Solanum xanthocarpum* Schrad. & Wendl have been used for centuries in South Asia, mainly India and Pakistan, for a wide range of ailments. However, the most important activities are its diuretic and lithotriptic effects according to their traditional usage and ancient literature.[14]

Pedalium murex Linn. belonging to the family of Pedaliaceae commonly known as 'Bada-gokhru' found in the sandy coast of Gujarat and in peninsular India.[15] As per indigenous system of medicine fruits of *P. murex* are reported to be useful in the treatment of wide range of an ailment, including urolithiasis.[14] The fruits as well as the leaves and stems produced milk mucilage when agitated, and it is recommended as a treatment for gonorrhea.[16] An infusion or extract prepared from leaves is diuretic and demulcent, useful in treating disorders of the urinary system such as ardor urine, dysuria, spermatorrhoea, and incontinence of urine. The aqueous extract of the whole plant has been found to possess analgesic and anti- inflammatory properties.[17] Extensive

phytochemical investigations on the plant have revealed the presence of pedalitin and pedalin (major flavonoids) along with piosmetin, dinatin, dinatin-7-glucoronide, quercetin, quercimeritin, quercetin-7- glucorhamnoside etc.[18] Although the plant contains several phytoconstituents, they have not been evaluated for their pharmacological activities in detail.

Solanum xanthocarpum Schrad. & Wendl. (Solanaceae) commonly known as Yellow Berried Nightshade (syn: kantakari), is a prickly diffuse bright green perennial herb, woody at the base, 2‑3m height found throught India, mostly in dry places as a weed on roadsides and waste lands. The fruits are glabrous, globular berries, green and white strips when young but yellow when mature.[19] The fruits are known for several medicinal uses like anthelmintic, antipyretic, laxative, antiinflammatory, antiasthmatic and aphrodisiac activities.[16] Chauhan et al.,[20] reported people of Muzaffarnagar (India) used *S. xanthocarpum* for the treatment of urinary track and kidney stone. The fruits are reported to contain several steroidal glycol-alkaloids like solasodine, solanacarpine, solanacarpidine, solancarpine, solasonine and solamargine.[21]

With light of above-mentioned pharmacological properties due to presence of active phytoconstitients, the purpose of this dissertation is to investigate the beneficial effects of fruits of *P. murex* and *S. xanthocarpum* at different doses for the prevention of kidney stone formation by *in vitro* and *in vivo* models of urolithiasis. We hypothesize that *P. murex* and *S. xanthocarpum* could inhibit the formation of kidney stones.

2. MATERIALS AND METHODS

2.1. Collection and identification of plant materials

P. murex fruits were collected from the coastal region of Surat, Guajrat during the month of August-September 2009 and *S. xanthocarpum* fruits were collected from Surat, Gujarat, during the month of April–May 2009. They were identified and authenticated by Prof. Manoo Parabia, Head of the Department of Bioscience, Veer Narmad South Gujarat University, Surat, India. Voucher specimens of the *S. xanthocaprum* and *P. murex* were deposited in the herbarium with no. PKP/10102008/1 and PKP/10102008/2 respectively.

2.2. Preparation of extracts

P. murex and *S.* xanthocarpum fruits were a shade dried and ground to coarse powder. Every time a new powder was packed in a Soxhlet's column and extracted with petroleum ether, ethyl acetate, methanol and water in their increasing polarity at different temperature for 24 h. The extraction procedure repeated, pooled extract was evaporated at 45°C, under vacuum and stored in an airtight container.

2.3. Qualitative phytochemicals analysis

The extracts were screened for the presence of various constituents (alkaloids, saponins, tannins, anthraquinones, sterol, flavonoids, terpenoids, glycosides, simple sugars) using standard protocol.[22]

2.4. Quantitative phytochemicals analysis

Spectrophotometric methods for total phenolic content and total flavonoid content while chromatographic methods i.e. HPTLC were used for determination of phytoconstituents in extracts with highest activity of *P. murex* and *S. xanthocarpum.*

2.5. Preparation of saponin rich extract

Saponin rich extract were prepared according to Hassan et al.[23] briefly, powder of *P. murex* fruits were extracted with methanol-water (1:1, v/v) by Soxhlet's column, pooled extracts were evaporated to one-third of initial volume. Remaining aqueous extract was partitioned with butanol (1:1, v/v) overnight at room temperature using separating funnel. Upper butanol extract was collected and lower aqueous layer further partitioned with butanol to increase the yield of crude saponin extracts. Butanol extracts were pooled and

evaporated to dryness. Same procedure was followed for the fruits of *S. xanthocarpum*.

2.6. Quantitative chromatographic analysis of saponin rich extracts

Quantitative chromatographic methods i.e. HPTLC were used to for determination of diosgenin and solasodine concentration in saponin rich extracts of *P. murex* and *S. xanthocarpum* respectively.

2.7. Residual solvents estimation in methanol and saponin rich extracts

Methanol and butanol are class II and III residual solvents according to United States Pharmacopoeia respectively. Therefore, both the solvents were determined according to United States Pharmacopoeia using gas chromatography.[24]

2.8. Animal

Adult albino Wistar rats (male for antiurolithiatic study) and female (for acute oral toxicity study) weighing between 200-250 g housed in standard conditions of temperature (22 ± 2°C), relative humidity (55 ± 5%) and light (12 h light-dark cycles) were used. They have been fed with standard pellet diet and water *ad libitum*. The study protocols were approved by the Institutional Animal Ethics Committee according to the regulation of Committee for the Purpose of Control and Supervision of Experiments on Animals.

2.9. Determination of maximum tolerable dose

The acute oral toxicity study was carried out in female rats as per the guideline set by the Organization for Economic Cooperation and Development (OECD) number 425 (up and down procedure) and found out the maximum tolerable dose.

2.10. Preliminary pharmacological evaluation

Pt. ether, ethyl acetate, methanol and water extracts of *P. murex* and *S. xanthocarpum* were evaluated in ethylene glycol induced nephrolithiasis in rats to find out extract of highest activity. The extract with highest activity was further used for detail study of antiurolithiatic activity using *in vitro* and *in vivo* models.

2.11. *In vitro* antiurolithiatic activity

In vitro antiurolithiatic activity of methanol extracts and saponin rich extracts of *P. murex* and *S. xanthocarpum* was performed according to Atmani and Khan[25] and find out

the effects of plants extracts and saponin rich extracts on nucleation and aggregation of calcium oxalate crystals formation.

2.12. *In vivo* antiurolithiatic activity

2.12.1. Ethylene glycol induced nephrolithiasis

An ethylene glycol model was used to induce urolithiasis.[13] The animals were randomly divided into nine groups containing six in each. Group I served as a vehicle-treated control and maintained on regular rat food and drinking water *ad libitum*. All remaining groups received calculi-inducing treatment for 28 days, comprised of 0.75% v/v ethylene glycol in drinking water *ad libitum*. Group II received ethylene glycol only served as a model control for 28 days. Group III was administered Cystone 750 mg/kg, p.o., body weight (The Himalaya Drug Company, India.) from 1st day to 28th day of calculi induction and served as a standard. Groups IV-IX served as treatment groups, received extract of *P. murex* (group IV, V, and VI) and *S. xanthocarpum* (group VII, VIII and IX) at doses of 100, 200, and 400 mg/kg, p.o., respectively, from 1st day to 28th day of calculi induction. Extract and standard drugs were dissolved in distilled water and given once daily by the oral route using the gastric tube.

Similar groups distribution was done with saponin rich extract of *P. murex* and *S. xanthocarpum* at different doses.

2.12.1.1. Collection and analysis of the urine

All animals were kept in individual metabolic cages, and 24 hrs urine samples were collected on 28th day of calculi induction treatment. Following volume, pH, and crystalluria determination, urine was acidified with a drop of concentrated HCl and stored at –20 °C for determination of various biochemical parameters. Urine was analyzed for calcium,[26] oxalate,[27] magnesium,[28] phosphate,[29] uric acid,[30] and citrate.[31]

2.12.1.2. Serum analysis

After the experimental period, blood was collected from retro-orbital under light ether anesthesia, and animals were killed by the cervical decapitation. Serum was separated by centrifugation at 10,000 × *g* for 10 min and analyzed for the creatinine, uric acid, urea, and blood urea nitrogen (BUN) using diagnostic kits (Span Diagnostics Ltd., India).

2.12.1.3. Kidney histopathology

The abdomen was incised and opened, then to remove both kidneys from each animal; isolated kidneys were cleaned off extraneous tissue, after that weighed and rinsed with ice-cold normal saline. The left kidney was fixed with 10% v/v neutral formalin and processed through graded alcohol series and xylene, embedded in paraffin, sectioned at 5 µm, and stained with the hematoxylin and eosin for histopathological examination under a light microscope and polarized microscope.

2.12.1.4. Kidney homogenate analysis

The right kidney was finely chopped and 20% homogenate prepared in Tris–HCl buffer (pH 7.4). Total kidney homogenate was used for assaying tissue calcium and oxalate, malondialdehyde (MDA), catalase, and reduced glutathione (GSH) measured.

2.12.2. Calcium oxalate seeds induced urolithiasis

Urolithiasis was induced by introducing a CaOX seed into the bladder of adult male albino Wistar rats.[32] The animals were randomly divided into nine groups containing six in each. Group I served as a vehicle-treated control and maintained on regular rat food and drinking water *ad libitum*. Group II as model control in which only CaOX seeds were implanted while group III as sham operated in which only surgical procedure was performed without implantation of CaOX seeds. Group IV was treated with Cystone (750 mg/kg, p.o.) for 14 days; followed by CaOX seeds implantation. Groups V-X served as treatment groups, received extract of *P. murex* (group IV, V, and VI) and *S. xanthocarpum* (group VII, VIII and IX) at doses of 100, 200, and 400 mg/kg, p.o., respectively, from 1st day to 14th day of CaOX seed implantation.

Same groups distribution was done with saponin rich extract of *P. murex* and *S. xanthocarpum*.

Concisely, rats under anesthesia (Ketamine 70 mg/kg, i.p., Diazepam 4 mg/kg, i.m.) had their bladder exposed through a suprapubic incision and a CaOX crystal (seed) of ~3 mm in diameter was introduced into the bladder. After suturing the bladder, muscle and skin, the animals were maintained in individual cages for 24 hrs for observation. Sham-operated animals underwent the same protocol, but the CaOX seed was not placed into the bladder. All animals were allowed free access to water *ad libitum*.

2.12.2.1. Preparation of CaOx seeds for implantation

To prepare the CaOX crystal, small disks of CaOX were obtained by a supersaturation reaction, as described previously.[32] Briefly, 100 ml of calcium chloride (0.4 mol/l) and 100 ml of potassium oxalate (0.4 mol/l) were mixed together by constant drop-wise addition to 300 ml of distilled water for 2 h with shaking at 75°C. The mixture was maintained under agitation at 75°C for an additional 5 h. crystals were washed and maintained in a hotplate at 37°C for two weeks to allow aggregation and growth of the seed. The resultant material was transferred to a template containing cylinders of 3 mm diameter to obtain small disks of CaOX. The crystals, which were rigid but not brittle, will be used for implantation. Disks were weight and sterilized before use.

2.12.2.2. X-ray examination

X-ray of the pelvic region was taken next day after the implantation of CaOX seeds (before drug administration) and after completion of the drug regimen, i.e. on 14th day two hrs after the drug administration, to determine the change in mass of implanted stone.

2.12.2.3. Urine analysis

All animals were kept in individual metabolic cages (Inco. Instruments and chemicals Pvt. Ltd., Ambala, India) and 24 h urine samples were collected on 14th day of CaOX induction and drugs treatment. Following volume determination, urine was acidified with a drop of concentrated HCl and stored at -20˚C for determination of various parameters. Urine was analyzed using commercially available kits for calcium,[26] uric acid,[30] creatinine and magnesium while oxalate and citrate were analyzed according to Hodgkinson et al.,[27] and Rajagopal[31], respectively.

2.12.2.4. Serum analysis

After the experimental period, blood was collected from retro-orbital under anesthetic conditions. Serum was separated by centrifugation at 10,000× g for 10 min and analyzed using commercially available kits for calcium, uric acid, creatinine, BUN (Span Diagnostics Ltd., Surat, India) while oxalate was analyzed according to Hodgkinson et al. 1972.

2.12.2.5. SEM of CaOX seeds

All animals were sacrificed; stones were dry and again weight for the change in mass, then were subject to scanning electron microscopy (SEM) for surface study.

2.12.3. Pharmacological activity of saponin rich fraction

2.12.3.1. Carrageenan-induced rat pedal inflammation

P. murex and *S. xanthocarpum* saponin rich fraction was evaluated for anti-inflammatory activity using the carrageenan-induced paw edema assay in rats according to Winter et al.[33] Rats were fasted for 12 hrs and were divided into eight groups and the effect of oral administration of *P. murex* at the dose of 20, 40, and 80 mg/kg while *S. xanthocarpum* at doses of 10, 20, and 40 mg/kg, indomethacin (10 mg/kg, p.o.) given as a 60 min pretreatment was studied. Paw edema was induced by sub-plantar injection of 100 µl of a 1% sterile carrageenan lambda in distilled water into the right hind paw. Paw volume was determined immediately before carrageenan injection and at selected times thereafter using a plethysmometer. The edema component of inflammation was quantified by measuring the increase in paw volume (ml) at before carrageenan injection and at 1, 2, 3, 4, 5 hrs after carrageenan injection with respect to the pre-injection value for each animal. The percentage inhibition of paw oedema in treated groups was then calculated by using the formula:

Percentage inhibition = (1 – Vt/Vc) X 100

Where Vt = is the oedema volume in the drug treated

Vc = is the oedema volume in the control group

2.12.3.2. Diuretic activity

The method described Wiebelhaus et al. 1965 was employed, with modification, for the assessment of diuretic activity. Healthy albino rats of either sex (160-200 g) were divided into eight groups of six animals each. They were fasted 18 hrs prior to the test, with free access to water. On the day of the experiment, animals were given 25 ml/kg of body weight normal saline orally. Group I received vehicle and served as control group. Group II Furosemide 100 mg/kg, Group III-V received saponin rich extract of *P. murex* at doses of 20, 40, and 80 mg/kg and group VI-VIII received saponin rich extract of *S. xanthocarpum* at the doses of 10, 20, and 40 mg/kg. All drugs/vehicle were administered orally (p.o.). Immediately after dosing, the rats were placed in the metabolic cages with special provision to collect faeces and urine. Animals were kept at room temperature of

35 ± 1^0C throughout the experiment. The observed parameters were total volume, Na^+, and K^+ excreted in the urine. Urine excreted for the next 5 hrs was collected and the total 5 hrs urine volume for each rat was compared with the volume of urine produced after the administration of normal saline.

2.12.3.3. Spasmolytic activity

Overnight fasted rats of either sex weighing 200-250 gm were sacrificed using ether anesthesia. Ileum was quickly dissected out and mounted in organ bath containing 25 ml Tyrode's solution maintained at 37 ± 1°C under basal tension of 500 mg. The composition of solution in mM was NaCl, 137; $CaCl_2$, 1.8; KCl, 2.7; glucose, 5.55; $NaHCO_3$, 11.9; $MgCl_2$, 1; NaH_2PO_4, 0.4. The solution continuously bubbled with air. The responses to drugs were recorded using isotonic frontal writing lever. The tissue was allowed to equilibrate for 30 min., during which the bathing solution was changed at every 10 min. The contractile responses of ileum to acetylcholine and $BaCl_2$ as agonists were recorded. The effects of varying doses of saponin rich extracts (1 to 8 mg /ml) of test drugs and their interaction with contractile responses of above mentioned agonists on ileum were studied.

2.12.3.4. In vitro anti-oxidant activity

2.12.3.4.1. DPPH radical scavenging activity

The free radical scavenging activity of saponin rich fraction of *P. murex* and *S. xanthocarpum* was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH.[34] DPPH solution (0.1 mM) in ethanol was prepared and 1 ml of this solution was added to 3 ml of extract solution in water at different concentrations (10-300 µg/ml). After 35 min, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\%\ \text{DPPH scavanged} = \frac{A(\text{cont}) - A(\text{test})}{A(\text{cont})} \text{ X } 100$$

where A(cont) is the absorbance of the control reaction and A(test) is the absorbance in the presence of the sample of the extracts.

2.12.3.4.2. Metal chelating activity

The chelating of ferrous ions by saponin rich extracts of *P. murex* and *S. xanthocarpum* was estimated by the method of Dinis et al..[35] Briefly, the extract samples (250 µl) were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), the mixture was shaken vigorously, and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as mg EDTA equivalent/g extract.

2.12.3.4.3. H_2O_2 radical scavenging activity

The ability of extracts to scavenge H_2O_2 was determined according to the method of Ruch et al.[36] A solution of H_2O_2 was prepared in phosphate buffer (pH 7.4). Extracts (10-300 µg/ml) in distilled water were added to a H_2O_2 solution (0.6 ml, 40 mM). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing the phosphate buffer without H_2O_2. The % of H_2O_2 scavenging of both the extracts and standard compounds was calculated as in the case of DPPH.

2.12.3.4.4. Reducing power

The reducing power of the extracts was determined according to the method of Oyaizu, 1986. Various concentrations of the saponin rich fraction of *P. murex* and *S. xanthocarpum* and Ascorbic acid (25-300 µg/ml) in 1.0 ml of de ionized water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5ml). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml,10%) were added to the mixture, which was then centrifuged at 1000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared $FeCl_3$ solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of their action mixture indicated increased reducing power.

2.13. Statistical analysis

The results were presented, as mean ± SEM. Difference among data was statistically analyzed using ANOVA followed by Dunnett's test to find out the level of significance using Sigmaplot software. Differences between the data were considered significant at $p < 0.05$.

3. RESULT AND DISCUSSION

3.1. Characteristics and qualitative phytochemical analysis of prepared extracts

Therapeutic efficacies of medicinal plants are attributed to the quality and quantity of active principles they contain. Alterations in these phytochemicals may vary its therapeutic effect and hence qualitative and quantitative phytochemical analysis of plant material used in this study was done with various methods like chemical reaction with different reagent for qualitative determination and spectrophotometric method and chromatographic method using HPTLC for quantitative estimation.

Characteristics and qualitative phytochemical analysis of petroleum ether, ethyl acetate, methanol and water extract of *P. murex* and *S. xanthocarpum* given in table 1 and table 2 respectively.

Table 1: Characteristics and qualitative phytochemical analysis of prepared extracts from *Pedalium murex*.

Plant constituents	**Petroleum ether extract**	**Ethyl acetate extract**	**Alcoholic extract**	**Aqueous extract**
% Yield (w/w)	2.6 %	4.3 %	7.6 %	9.3 %
Color	Greenish brown	Reddish brown	Blackish brown	Blackish brown
Consistency	Semi solid & oily	Semi solid & sticky	Dry powder	Dry powder
Alkaloids	-	+	++	+
Carbohydrates	-	-	+	++
Glycosides	-	+	++	+
Saponin	-	++	+++	+++
Phenolic & Tannins	-	+	++	+
Flavonoids	-	++	+++	+
Proteins & amino acids	-	-	+	++
Fixed oils and fats	++	+	++	-

(**-** = absent, **+** = present, **++** = moderately present, **+++** = abundantly present)

3.2. Quantitative phytochemical analysis

Total phenolic content and total flavonoid content of methanol extract of both

plants shown in table 3.

Table 2: Characteristics and qualitative phytochemical analysis of prepared extracts from *Solanum xanthocarpum*.

Plant constituents	**Petroleum ether extract**	**Ethyl acetate extract**	**Alcoholic extract**	**Aqueous extract**
% Yield (w/w)	1.64 %	2.69 %	13.66 %	20.2 %
Color	Brown	Yellowish brown	Reddish brown	Reddish brown
Consistency	Semi solid & oily	Semi solid & sticky	Slight sticky powder	Dry powder
Alkaloids	-	+	+++	++
Carbohydrates	-	-	+	+
Glycosides	-	-	+	+
Saponin	-	++	+++	+++
Phenolic & Tannins	-	++	+++	+
Flavonoids	-	+	++	+
Proteins & amino acids	-	-	+	++
Fixed oils and fats	+	-	++	-

(**-** = absent, + = present, ++ = moderately present, +++ = abundantly present)

Table 3: Total phenolic and total flavonoid content of methanol extract of *P. murex* and *S. xanthocarpum.*

Methanolic extract of plants	**Total phenolic content** (mg of gallic acid equivalent/g of dry extract)	**Total flavonoid content** (mg of rutin equivalent/g of dry extract
P. murex	3.46 ± 0.23	15.72 ± 1.03
S. xanthocarpum	11.73 ± 0.96	10.97 ± 0.82

Chromatographic technique by HPTLC showed the presence of diosgenin and solasodine in methanolic extract of *P. murex* and *S. xanthocarpum* respectively.

Table 4: Characteristics and qualitative phytochemical analysis of saponin rich fraction.

Plant constituents	***P. murex***	***S. xanthocarpum***
% Yield (w/w)	1.24 %	3.28 %
Color	Brownish black	Orange -red
Consistency	Hygroscopic powder	Dry powder
Alkaloids	+	+++
Carbohydrates	+	+
Glycosides	+	+
Saponin	+++	+++
Phenolic & Tannins	-	-
Flavonoids	+	-
Proteins & amino acids	-	-
Fixed oils and fats	-	-

(- = absent, + = present, ++ = moderately present, +++ = abundantly present)

3.3. Quantitative chromatographic analysis of saponin rich extracts

The concentration of diosgenin in *P. murex* and solasodine in *S. xanthocarpum* by using HPTLC was found to be 0.032 % w/w and 0.658 % w/w respectively.

3.4. Residual solvents estimation in methanol and saponin rich extracts

According to USP and ICH guideline methanol is class 2 residual solvent (minimum limit <3000 ppm) which is solvents to be limited as non-genotoxic animal carcinogens or possible causative agents of other irreversible toxicity, such as neurotoxicity or teratogenicity while n-butanol is class 3 residual solvents (minimum limit < 0.05 %) which is solvents with low toxic potential humans; no health-based exposure limit is needed.[24]

Table 5: The residual solvents in methanolic and saponin rich extracts of *P. murex* and *S. xanthocarpum*.

Plants	**Methanolic extract**	**Saponin rich fraction**	
	Methanol (ppm)	**Mehtanol (ppm)**	**n-Butanol (%)**
P. murex	2267.56	97.05	0.272
S. xanthocarpum	1575.03	39.71	0.324
Minimum USP limits	<3000	<3000	<0.5

3.5. Determination of maximum tolerable dose

Safety should be the overriding criterion in the selection of medicinal plants for use in health care systems. Methanolic extract of *P. murex* and *S. xanthocarpum* did not show any mortality up to dose of 2000 mg/kg and were considered as safe. From which 200 mg/kg, p.o. was selected for preliminary pharmacological evaluation for crude extracts prepared using petroleum ether, ethyl acetate, methanol and water from the *P. murex* and *S. xanthocarpum*. Three different experimental doses 100, 200 and 400 mg/kg of methanolic extracts were selected for the main study from maximum tolerable dose and also by considering previously published literatures of *P. murex* [37, 38] and *S. xanthocarpum*.[39, 40]

Saponin rich fraction of *P. murex* at 1000 mg/kg and *S. xanthocarpum* at 800 mg/kg did not show any mortality and were considered as safe. Two different experimental doses for *P. murex* were 40 mg/kg and 80 mg/kg while for *S. xanthocarpum* were 20 mg/kg and 40 mg/kg selected from the results of dose fixation study for main study.

3.6. Preliminary pharmacological evaluation

Variations in quantity of extract in specific solvent can be attributed to number of contents present, polarity of contents, properties of solvent used and varying pH and temperature at the time of extraction. Therefore, it is necessary to find the extract with highest therapeutic activity in animals using preliminary pharmacological evaluation of all extracts prepared from different solvents from which detailed study was performed to establish possible mechanism of action of extract with highest activity.

From preliminary pharmacological evaluation, it was found that methanolic extract of *P. murex* and *S. xanthocarpum* showed highest antiurolithiatic activity. Therefore, methanolic extracts of both plants were selected for main study using *in vitro* and *in vivo* model for evaluation of antiurolithiatic activity.

3.7. Evaluation of antiurolithiatic activity by *in vitro* model

In this work, we performed an *in vitro* crystallization study enabling the specification of kinetic and thermodynamic conditions of formation, growth and aggregation of CaOx stone. The slow and controlled diffusion of species to the growing crystals is very useful to study the growth and inhibition of CaOx crystals *in vitro*. Different experimental procedure have been proposed using synthetic diluted or artificial

supersaturated aqueous solution of urine.[41]

3.7.1. Effects of methanolic extract on *in vitro* crystallization

The supersaturation of urine with CaOX, the most common component of kidney stones is an important factor in crystallization, with later factors being nucleation, growth and aggregation.[42]

3.7.1.1. Effect on nucleation assay

Nucleation is very important and first step for the initiation of crystals, than growth and aggregation.[25] The main findings of the present study were that extracts from plants inhibited the crystallization by inhibiting nucleation of CaOx in solution; there were less and smaller particles with increasing concentrations of methanolic extract of *P. murex* and *S. xanthocarpum* from 100 µg/ml to 1000 µg/ml as decreasing the absorbance at 620 nm. These results were confirmed in the nucleation assay, which showed that the extract contained nucleation-preventing agents.

3.7.1.2. Effect on aggregation assay

Aggregation may be an important factor in the genesis of stones.[43] Recurrent calcium stone formers excrete clusters of crystals in their urine, caused by aggregation, also named agglomeration, whereas urine from normal people contains mainly single crystals. *P. murex* and *S. xanthocarpum* fruit methanol extract showed, increases % inhibition of CaOx crystal aggregation in dose dependent manner at varying concentration from 100 µg/ml to 1000 µg/ml.

3.7.2. Effect of saponin rich fraction of on *in vitro* crystallization

3.7.2.1. Effect on nucleation assay

As discuss above that nucleation is very important initial steps involve for the development of CaOx crystals[25] which was inhibited by saponin rich fraction of *P. murex* and *S. xanthocarpum* at various concentration of 10 µg/ml to 100 µg/ml, which was showed by decreasing the absorbance at 620 nm.

3.7.2.2. Effect on aggregation assay

The limiting factors in stone formation could be those processes that affect the size

of the particles to from aggregation, because aggregated particles may become large enough to occlude the urinary tract, leading to stone formation.[44] *P. murex* and *S. xanthocarpum* fruit saponin rich fraction showed, increases % inhibition of CaOx crystal aggregation in dose dependent manner at varying concentration from 10 µg/ml to 100 µg/ml.

3.8. Evaluation of antiurolithiatic activity by *in vivo* model

Male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans and earlier studies shown that the amount of stone deposition in female rats was significantly less.[45] Evidence in previous studies indicated that in response to 28 day period of ethylene glycol (0.75%, v/v) administration, young male albino rats form renal calculi composed mainly of calcium oxalate.[46] The biochemical mechanisms for renal stones formation are related to an increase in the urinary concentration of oxalate and glycolic acid, the precursor of oxalic acid, is known to increase significantly the incidence of oxalate lithiasis.[13]

3.8.1. Effect of methanolic extract on ethylene glycol induced urolithiasis

3.8.1.1. Effect of methanolic extract on physical parameters

Loss weight observed in untreated group is due to anorexia due to disturbances in carbohydrates, proteins or fat metabolism, which is affected by the oxalate. Indeed the toxic nature of oxalic acid and its nephrotoxicity was well recognized in the nineteenth century.[47] Administration of ethylene glycol produced significant ($P < 0.001$) reduction in body weight in model control (-6.06 ± 0.84 %) as compared to normal control animals (13.42 ± 1.41 %). Significant ($P < 0.001$) improvement in body weight was observed in treated groups i.e. methanolic extract of *P. murex* and *S. xanthocarpum* at different doses in dose in dose dependent manner. Lithogenic treatment as ethylene glycol was significantly ($P < 0.001$) increased the water intake in model control group (31.52 ± 2.42) as compared to normal control group (7.16 ± 0.53). Treatment with *P. murex* and *S. xanthocarpum* at 200 mg/kg and 400 mg/kg significantly decreased water intake but not significant at 100 mg/kg as compared to model control group.

Increased urine output may have two effects. First, the mechanical diuresis that ensues may prevent urine stagnation and the formation of symptomatic calculi. More likely is that the dilute urine alters the supersaturation of stone components, which is one

of the favorable requisition factors for stone formation by crystallization.[48] The 24 hrs urine volume was high in model control group ($P < 0.001$) compared to those of the vehicle control animals. While in the CYSTONE, *P. murex* and *S. xanthocarpum* methanolic extract treated groups, urine volume was significantly higher than that of model control group and normal control.

At low urine pH (< 5.5), the undissociated form of uric acid predominates, leading to uric acid and/or calcium stone formation. Acidic urine is usually found in humans with idiopathic renal calcium oxalate stone formation, whereas chronic hyperoxaluric rats have alkaline urine.[49] Lithogenic treatment also changed urine pH in the untreated group and treated groups, but it was not to a significant extent at various doses of *P. murex* and *S. xanthocarpum* fruit methanolic extract and even with CYSTONE treatment.

3.8.1.2. Effect of methanolic extract on urinary parameters

Crystalluria could occur similarly in both healthy and stone forming individuals where the latter tend to excrete larger and aggregated particles than the formers.[50] Crystals were absent in urine of vehicle control animals, while in the lithogenic treatment group, crystals were more numbers with larger size. Treatment with *P. murex* and *S. xanthocarpum* fruit extract clearly reduced the crystal number as well as crystal size in dose dependent manner.

Hyperoxaluria is a more significant risk factor in the pathogenesis of renal stone. It has been reported that oxalate play an important role in stone formation and has about 15-fold greater effect than urinary calcium.[45] Uric acid and inorganic phosphate interferes with calcium oxalate solubility and it binds and reduces the inhibitory activity of glycosaminoglycans.[51] There was significant increased urinary excretion of calcium, oxalate, uric acid and phosphate ($P < 0.001$) in calculi induced group compared to vehicle control, which were significantly decreased in-group of animals treated with CYSTONE. However, co-treatment with methanolic extract of *P. murex* and *S. xanthocarpum* significantly prevented these changes in the urinary calcium, oxalate, uric acid, and phosphate excretion dose dependently compared to lithogenic group.

Hypocitraturia is the major metabolic abnormality in patients with renal stones. Investigations of citrate metabolism in stone formers have shown that tubular citrate reabsorption is the main mechanism regulating urinary citrate excretion.[52] Normal urine contains many inorganic and organic inhibitors of crystallization, magnesium is one such well-known inhibitors. Low levels of magnesium are also encountered in stone formers as

well as in stone-forming rats. The magnesium levels return to normal on drug treatment.[51] Urinary excretion of magnesium and citrate was decreased significantly ($P < 0.001$) in stone forming group compared to vehicle control. Supplementation with the CYSTONE significantly increases while methanolic extract of *P. murex* and *S. xanthocarpum* substantially prevented these changes in citrate dose dependently but significant increase in magnesium level was achieved at higher doses only.

3.8.1.3. Effect of methanolic extract on serum parameters

Markedly high levels of urinary oxalate ensue, leading to increased saturation of calcium oxalate, aggressive stone formation, and marked nephrocalcinosis.[53] High urinary calcium concentrations lead to increased urinary saturation of calcium salts and reduced urinary inhibitory activity by way of complexation with negatively charged inhibitors such as citrate.[54] There was significant increase in serum calcium and oxalate level observed in model control group as compared to normal control. Treatment with the CYSTONE significantly decreases in serum and calcium level compared to model control. Treatment with *P. murex* and *S. xanthocarpum* decreases serum calcium level but not to a statistically significant at given doses. While simultaneous administration of methanolic extract of *P. murex* and *S. xanthocarpum* significantly decreased serum oxalate level at all the given doses.

As compared to a vehicle control group, the stone inducing regimen caused a significant impairment of renal functions of the untreated group shown by significant high level ($P < 0.001$) of creatinine, uric acid, urea and BUN in serum. These were dose dependently inhibited in the animal receiving a simultaneous treatment with CYSTONE, and methanolic extract of *P. murex* and *S. xanthocarpum*.

3.8.1.4. Effect of methanolic extract on kidney homogenate parameters

Nephrolithiasis can leads to increase deposition of crystalline materials like oxalate and CaOx and inflammatory reaction caused by oxalate increased the weight of wet kidney.[46] Weight of the kidney was notably ($P < 0.01$) increased in the lithogenic group compared to vehicle control. Treatment with *P. murex* and *S. xanthocarpum* significantly decreased the kidney weight in a dose dependent manner compared to calculi induced group. The crystalline components like calcium and oxalate were significantly increased in the kidney of stone forming rat compared to control ($P < 0.001$). Treatment of plants extract significantly reduced the renal content of these stone forming constituents in the

treated group.

Stone inducing treatment caused hypertrophy and extensive CaOx crystal deposition in kidneys of untreated rats accompanied by oxidative damage as reflected from increased levels of markers of oxidative injury: MDA and decreased activities of antioxidant enzymes and GSH levels in kidneys as well as deteriorated renal functions.[46] Stone inducing treatment causes oxidative cell injury indicated by enhanced MDA ($P < 0.001$) content and decreased GSH ($P < 0.001$) level as well as activities of antioxidant enzyme catalase ($P < 0.001$) in kidney of untreated rats compared to control animals. A co-administration with *P. murex* and *S. xanthocarpum* protected against the oxidative changes induced by lithogenic treatment in the dose dependent manner.

When observed under polarized light microscope, many birefringent crystalline deposits in the histological preparations were seen in tubules of all regions of kidneys: cortex, medulla and papilla, of all the animals in the untreated i.e. model control group. In methanolic extract of *P. murex* and *S. xanthocarpum* treated groups, such deposits were visibly small and less abundant dose dependent manner as compare to those in the model control kidneys

Histopathological examination revealed the normal glomeruli and tubular region with the absence of CaOX crystal in vehicle control animal. While in stone forming group severe glomeruli damage, RBCs deposition, which leads to hematuria, numerous and large size CaOX crystal deposition in renal tubule and dilation of the proximal tubules with interstitial inflammation ware observed in the renal tissue. Treatment with *P. murex* and *S. xanthocarpum* fruit extracts decreased glomeruli damage, less RBCs accumulation, fewer numbers and smaller size CaOX crystal deposition with fragmentation in the dose dependent manner in comparison to calculi induced group.

3.8.2. Effect of methanolic extract on calcium oxalate seeds induce urolithiasis

3.8.2.1. Effect of methanolic extract on physical parameters

As mentioned above that lithogenic treatment affects the carbohydrate, protein and fat metabolism that are responsible for weight loss.[47] After two weeks of insertion of stone in the bladder, there was significant ($P < 0.001$) reduction in body weight of model control (-1.43 ± 0.08) as compared to normal control (4.19 ± 0.39) animals. Reduction in body weight was also observed in treatment with methanolic extract of *P. murex* and *S. xanthocarpum* at lower doses, but rather than reduction treatment with CYSTONE ($5.43 \pm$

0.46), *P. murex* and *S. xanthocarpum* at higher doses improve the weight as compared to model control. Average daily water intake was significantly increased in model control (12.04 ± 1.07 ml) animals as compared to normal (5.47 ± 0.41 ml). Whereas reduction in water intake was observed with the treatment of CYSTONE (7.02 ± 0.53 ml), methanolic extract of *P. murex* as compared to model control. There was no significant difference in water intake of animals in sham operated groups (6.1 ± 0.43 ml) as compared to normal control.

Decreasing the urinary output can leads to supersaturation, which trigger the crystallization process.[48] Dilution of urine will decreases the supersaturation and thereby decreases the risk of stone development. The urinary output was increased significantly ($P < 0.01$) in CaOx seeds induced rats as compared to vehicle treated group was 15.96±0.62 ml/day/rat on the 14th day. Treatment with CYSTONE and methanolic extracts of *P. murex* at 400 mg/kg and *S. xanthocarpum* at 200 mg/kg and 400 mg/kg the urine output was higher than that of the calculi induced groups but statistical significant only at *S. xanthocarpum* at 400 mg/kg while the urinary pH remain unaffected in group of treated animals.

3.8.2.2. Effect of methanolic extract on urinary parameters

Urinary elevated levels of calcium and oxalate in the model control group are important for an aetiological point of view, as calcium ions form calcium oxalate and other insoluble salts, which tend to be crystallized.[55] There was a significantly increased oxalate and Calcium concentration ($P < 0.001$) of the urine collected from the model control group as compared to control group. Treatment with CYSTONE and methanolic extract of *P. murex* at 200 mg/kg and 400 mg/kg and *S. xanthocarpum* at all doses prevented the change in urinary oxalate and calcium contents.

Low levels of magnesium and citrate are also encountered in stone formers as well as in stone-forming rats. Their levels return to normal on drug treatment.[46] Level of urolithiasis inhibitors like magnesium and citrate was significantly decreased in model control group as compared to normal control in urine after 14th days of implantation with CaOx seed in bladder. Treatment with CYSTONE improves the level of magnesium and citrate significantly as compared to model control group. Supplementation with methanolic extract of *P. murex* at 200 mg/kg and 400 mg/kg while *S. xanthocarpum* at all doses increased the level of citrate in urine as compared to model control dose dependently. Treatment with methanolic extract of *P. murex* increased the level of

magnesium but not statistical significant when compared with model control group while *S. xanthocarpum* significantly increased the level of magnesium at higher doses.

Uric acid interferes with CaOx solubility, and it binds and reduces the inhibitory activity of glycosaminoglycans which promotes stone formation.[13] Level of uric acid in urine was significantly increased in group of animals of model control compared to normal control and sham operated. Treatment with CYSTONE significantly decreased the level of urinary uric acid as compared to model control in CaOx seeds induced urolithiasis. Administration with methanolic extract of *P. murex* and *S. xanthocarpum* significantly decreased uric acid level in urine at higher doses.

3.8.2.3. Effect of methanolic extract on serum parameters and % increase in stone matrix

Generally, serum calcium and oxalate level is increased in metabolic defect.[4] There was no significant changed in level of serum calcium and oxalate observed after 14 days of CaOx seeds implantation in rat bladder of model control group as compared to normal control. Treatment with CYSTONE, methanolic extract of *P. murex* and *S. xanthocarpum* did not caused any significant changes in serum calcium and oxalate level when compared with model control group.

Lithogenic treatment causes renal impairment that was indicated by significant increasing the serum creatinine and BUN level in model control as compared to normal control group. Treatment with CYSTONE, methanolic extract of *P. murex* and *S. xanthocarpum* improve the renal function, which was shown by significant decreased in serum creatinine level in dose dependent manner. BUN was significantly decreased by the treatment with *P. murex* at 400 mg/kg but not at lower doses while *S. xanthocarpum* had significant effect at higher doses.

CaOx implantation can causes seed induced crystallization which is responsible for increases in stone matrix. Implantation of calcium oxalate crystals in the urinary bladder of rats induced growth of urinary stones after 14 days of surgery. Percentage increase in stone was very high while treatment with CYSTONE, methanolic extract of *P. murex* and *S. xanthocarpum* significantly prevented the increase in stone matrix in dose dependent manner. Surface was performed on recovered stones 14 days after the CaOx seeds implantation using scanning electron microscopy. It was observed that stones recovered from model control group shows rough and uniform surface without crack while treatment groups shows rough surface with crack which are clearly visible at high resolution

compared to model control group which indicated that treatment with CYSTONE, methanolic extract of *P. murex* and *S. xanthocarpum* has capacity to break implanted stones.

3.8.3. Effect of saponin rich fraction on ethylene glycol induced urolithiasis

3.8.3.1. Effect of saponin rich fraction on physical parameters

Loss weight observed in untreated group is due to anorexia due to disturbances in carbohydrates, proteins or fat metabolism, which is affected by the oxalate.[47] Administration of ethylene glycol produced significant ($P < 0.001$) reduction in body weight in model control (-4.53 ± 0.62 %) as compared to normal control animals (11.29 ± 1.40 %). Significant ($P < 0.001$) improvement in body weight was observed in treated groups i.e. saponin rich fraction of *P. murex* and *S. xanthocarpum* at different doses in dose dependent manner. Lithogenic treatment as ethylene glycol was significantly ($P < 0.001$) increased the water intake in model control group (34.17 ± 2.82 ml) as compared to normal control group (10.03 ± 0.94 ml). Treatment with saponin rich fraction of *P. murex* at 80 mg/kg and *S. xanthocarpum* at 20 mg/kg and 40 mg/kg significantly decreased water intake when compared with model control group.

Increased urine output may have two effects. First, the mechanical diuresis that ensues may prevent urine stagnation and the formation of symptomatic calculi. More likely is that the dilute urine alters the supersaturation of stone components, which is one of the favorable requisition factors for stone formation by crystallization.[48] The urinary output was increased in calculi induced rats as compared to vehicle treated group on the 28^{th} day but not to statistically significant. Treatment with saponin rich fraction of *S. xanthocarpum*, the urine output was significantly increased when compared with calculi induced groups in dose dependently. Lithogenic treatment also causes significantly increased in urine pH in the model control group than control group. Administration with CYSTONE, saponin rich fraction of *P. murex* and *S. xanthocarpum* significantly decreases the urine pH.

3.8.3.2. Effect of saponin rich fraction on urinary parameters

Crystalluria could occur similarly in both healthy and stone forming individuals where the latter tend to excrete larger and aggregated particles than the formers.[50] The analysis of crystalluria showed that CaOx crystals were absent in control rats while

lithogenic rats excreted more CaOx crystals which were larger than in treated rats i.e. CYSTONE and saponin rich fraction of *P. murex* and *S. xanthocarpum* in dose dependent manner.

Hyperoxaluria is a more significant risk factor in the pathogenesis of renal stone. It has been reported that oxalate play an important role in stone formation and has about 15-fold greater effect than urinary calcium.[45] Uric acid and inorganic phosphate interferes with calcium oxalate solubility and it binds and reduces the inhibitory activity of glycosaminoglycans.[51] In parallel with crystalluria, there was an increased oxalate and Calcium concentration ($p < 0.001$) of the urine collected from the model control group as compared to control group. Administration of saponin rich fraction of both these plants shows significant protective effect in ethylene glycol induced urolithiasis in dose dependent manner. Other changes in the urinary composition changes by lithogenic treatment were urinary phosphate and uric acid which was significantly increased in model control group as compared to normal control while treatment with saponin rich fraction of *P. murex* and *S. xanthocarpum* significantly decreased uric acid level in dose dependent manner as compared to model control group. On the other hand, *P. murex* significantly decreases phosphate level at all the doses gradually while *S. xanthocarpum* shows significant effect at dose of 80 mg/kg but not at 40 mg/kg as compared to model control group.

Hypocitraturia is the major metabolic abnormality in patients with renal stones. Investigations of citrate metabolism in stone formers have shown that tubular citrate reabsorption is the main mechanism regulating urinary citrate excretion.[52] Normal urine contains many inorganic and organic inhibitors of crystallization, magnesium is one such well-known inhibitors. Low levels of magnesium are also encountered in stone formers as well as in stone-forming rats. The magnesium levels return to normal on drug treatment.[51] Level of urolithiasis inhibitors like magnesium and citrate was significantly decreased in model control group as compared to normal control in urine after 28 days of administration with ethylene glycol. Supplementation with saponin rich fraction of *P. murex* and *S. xanthocarpum* significantly increased the level of magnesium and citrate in urine as compared to model control dose dependently.

Urinary glycosaminoglycan is one of the inhibitors of urolithiasis by decreasing the binding of calcium with oxalate thereby decreasing the crystallization.[13] Lithogenic treatment significantly ($P < 0.001$) decreased in model control (3.48±0.52) group as compared to normal control (10.38±1.34) while simultaneous treatment with saponin rich

fraction of *P. murex* was significantly ($P < 0.01$) increases GAGs at 80 mg/kg but not significant at 40 mg/kg while *S. xanthocarpum* significantly increases level of GAGs at given doses.

3.8.3.3. Effect of saponin rich fraction on serum parameters

Because of absent of metabolic defect in calcium and oxalate metabolism, amount of calcium and oxalate was measured in serum. There was significant increase in serum calcium and oxalate level observed in model control group as compared to normal control. Treatment with saponin rich fraction of *P. murex* and *S. xanthocarpum* decreases serum calcium level but not to a statistically significant at given doses while they significantly decreases the serum oxalate level dose dependently.

Lithogenic treatment causes renal impairment, which was indicated by significant increasing the serum creatinine, uric acid, urea and BUN level in model control as compared to normal control group. Treatment with CYSTONE, saponin rich fraction of *P. murex* and *S. xanthocarpum* improve the renal function, which was shown by significant decreased in serum creatinine, uric acid, urea and BUN level in dose dependent manner.

3.8.3.4. Effect of saponin rich fraction on kidney homogenate parameters

Lithogenic treatment increases inflammatory reaction in kidney due to CaOx crystal deposition, which can leads to increase the wet kidney weight. Administration of ethylene glycol significantly ($P < 0.001$) increased wet kidney weight in model control as compared to normal control. Treatment with saponin rich fraction of *P. murex* and *S. xanthocarpum* significantly ($P < 0.001$) decreases the wet kidney as compared to model control group.

Stone inducing regimen i.e. ethylene glycol increases the deposition of calcium and oxalate in kidney which is shown by significant ($P < 0.001$) increasing the amount of calcium and oxalate in kidney homogenate of model control as compared to normal control. Administration of saponin rich fraction of the *P. murex* and *S. xanthocarpum* also prevent the deposition of calcium and oxalate in kidney shown by significantly decreasing the amount in dose dependent manner.

Stone inducing treatment caused hypertrophy and extensive CaOx crystal deposition in kidneys of untreated rats accompanied by oxidative damage as reflected from increased levels of markers of oxidative injury: MDA and decreased activities of antioxidant enzymes and GSH levels in kidneys as well as deteriorated renal functions.[46]

Lithogenic treatment causes increasing the oxidative changes measured by level of malondialdehyde (MDA), reduced glutathione (GSH) and activity of catalase in kidney homogenate. Stone formation in kidney produced severe oxidative stress which was confirmed by significantly ($P < 0.001$) increases level of MDA, reduced activity of catalase and GSH as compared to model control group. Treatment with CYSTONE significantly decreases the CaOx induced oxidative changes indicated by decreasing the level of MDA and increasing the antioxidant enzyme catalase and GSH compared to model control. Administration of saponin rich fraction of *P. murex* and *S. xanthocarpum* significantly decreases the MDA level compared to model control in dose dependent manner while level of GSH significantly increases at all given doses of both plant. Activity of catalase was significantly increases in saponin rich fraction of *P. murex* and *S. xanthocarpum* compared to model control.

When observed under polarized light microscope, many birefringent crystalline deposits in the histological preparations were seen in tubules of all regions of kidneys: cortex, medulla and papilla, of all the animals in the untreated i.e. model control group. In saponin rich fraction of *P. murex* and *S. xanthocarpum* treated groups, such deposits were visibly small and less abundant in dose dependent manner as compare to model control kidneys.

No alteration was observed in the kidney of the normal control animals as well in the animals treated with CYSTONE. Severe renal damage was observed in model control group as glomerular congestion, peritubular congestion, epithelial desquamation, blood vessel congestion, inflammatory cells and crystal deposition. Treatment with saponin rich fraction of *P. murex* and *S. xanthocarpum* prevent these pathological changes at all administered doses.

3.8.4. Effect of saponin rich fraction CaOx seeds induced urolithiasis

3.8.4.1. Effect of saponin rich fraction on physical parameters

As mentioned above that lithogenic treatment affects the carbohydrate, protein and fat metabolism that are responsible for weight loss.[47] After two weeks of insertion of stone in the bladder, there was significant ($P < 0.001$) reduction in body weight of model control (-2.31±0.12) as compared to normal control (6.42±0.57) animals. Reduction in body weight was also observed in treatment with saponin rich fraction of *P. murex* at lower dose, but rather than reduction, treatment with CYSTONE, *P. murex* at 80 mg/kg

and *S. xanthocarpum* improve the weight as compared to model control. Average daily water intake was significantly increased in model control (14.86±1.32 ml) animals as compared to normal. Whereas significant reduction in water intake was observed with the treatment with saponin rich fraction of *P. murex* and *S. xanthocarpum* as compared to model control. There was no significant difference in water intake of animals in sham operated groups (6.59±0.63 ml) as compared to normal control.

Decreasing the urinary output can leads to supersaturation, which trigger the crystallization process.[48] Dilution of urine will decreases the supersaturation and thereby decreases the risk of stone development. The urinary output was increased significantly ($p < 0.01$) in CaOx seeds induced rats as compared to vehicle treated group was 13.34±0.76 ml/day/rat on the 14th day. Treatment with CYSTONE and saponin rich fraction of *P. murex* and *S. xanthocarpum* urine output was higher than that of the calculi induced groups but statistical significant only with CYSTONE. Lithogenic treatment also significantly increased in urine pH in the model control and sham operated group than normal control group, but it was not to a significant extent following the administration with CYSTONE and at the various doses of saponin rich fraction of *P. murex* and *S. xanthocarpum.*

3.8.4.2. Effect of saponin rich fraction on urinary parameters

Urinary elevated levels of calcium and oxalate in the model control group are important for an aetiological point of view, as calcium ions form calcium oxalate and other insoluble salts, which tend to be crystallized.[55] There was a significantly increased oxalate and Calcium concentration ($P < 0.001$) of the urine collected from the model control group as compared to normal control and sham-operated group. Treatment with CYSTONE and saponin rich fraction of *P. murex* at 80 mg/kg and *S. xanthocarpum* at all doses prevented the change in urinary calcium contents while oxalate content was significantly ($P < 0.05$) lower at higher doses of saponin rich fraction of *P. murex* and *S. xanthocarpum* as compared to model control group.

Low levels of magnesium and citrate are also encountered in stone formers as well as in stone-forming rats. Their levels return to normal on drug treatment.[46] Level of urolithiasis inhibitors like magnesium and citrate was significantly decreased in model control group as compared to normal control in urine after 14th days of implantation with CaOx seed in bladder. Supplementation with saponin rich fraction of *P. murex* and *S. xanthocarpum* at all doses increased the level of magnesium and citrate in urine as

compared to model control as dose dependently

Uric acid interferes with CaOx solubility, and it binds and reduces the inhibitory activity of glycosaminoglycans which promotes stone formation.[13] Level of uric acid in urine was significantly ($P < 0.001$) increased in-group of animals of model control compared to normal control and sham operated. Administration with saponin rich fraction of *P. murex* and *S. xanthocarpum* significantly ($P < 0.001$) decreased uric acid level in urine at all administered doses of both plants as compared to model control in CaOx seeds induced urolithiasis in bladder

Urinary glycosaminoglycan is one of the inhibitors of urolithiasis by decreasing the binding of calcium with oxalate thereby decreasing the crystallization.[13] Lithogenic treatment significantly ($P < 0.001$) decreased in model control group as compared to normal control and sham operated group while simultaneous treatment with CYSTONE significantly ($P < 0.001$) increased the level of GAGs. Administration with saponin rich fraction of *P. murex* was increases GAGs at 40 mg/kg and 80 mg/kg but not to a statistically significant while, *S. xanthocarpum* significantly increases level of GAGs at 20 mg/kg and 40 mg/kg as compared to model control group.

3.8.4.3. Effect of saponin rich fraction on serum parameters & % increase in stone matrix

As mentioned above that lithogenic treatment affects the carbohydrate, protein and fat metabolism that are responsible for weight loss.[47] There was no significant changed in level of serum calcium and oxalate observed after 14 days of CaOx seeds implantation in rat bladder of model control group as compared to normal control. Treatment with CYSTONE, saponin rich fraction of *P. murex* and *S. xanthocarpum* did not caused any significant changes in serum calcium and oxalate level when compared with model control group.

Lithogenic treatment causes renal impairment affect the glomerular filtration rate which was indicated by significant increasing the serum creatinine and BUN level in model control as compared to normal control group. Treatment with CYSTONE, saponin rich fraction of *P. murex* and *S. xanthocarpum* improve the renal function, which was shown by significant decreased in serum creatinine and BUN level in dose dependent manner.

CaOx implantation can causes seed induced crystallization which is responsible for increases in stone matrix. Implantation of calcium oxalate crystals in the urinary bladder

of rats induced growth of urinary stones after 14 days of surgery. Percentage increase in stone was very high in model control group while treatment with CYSTONE, saponin rich fraction of *P. murex* and *S. xanthocarpum* significantly prevented the increase in stone matrix.

Surface was performed on recovered stones 14 days after the CaOx seeds implantation using scanning electron microscopy (SEM). It was observed that stones recovered from model control group shows rough and uniform surface without crack while treatment groups shows rough surface with crack which are clearly visible at high resolution compared to model control group which indicated that treatment with CYSTONE, saponin rich fraction of *P. murex* and *S. xanthocarpum* has capacity to break implanted stones.

3.9. Evaluation of pharmacological activities of saponin rich fraction

Beneficial pharmacological activities that are helpful in prevention of urolithiasis like anti-inflammatory, diuretic, in vitro spasmolytic and in vitro antioxidant activity were evaluated using saponin rich fraction of *P. murex* and *S. xanthocarpum*.

3.9.1. Evaluation of anti-inflammatory activity of saponin rich fraction

It is well established that oxalate and CaOx crystals play very important role in causing inflammation in renal tissue.[47] Animals treated with saponin rich fraction of *P. murex* at 40 mg/kg showed significant reduction in paw volume at 4 hrs while 80 mg/kg showed significant reduction from 2 hrs to 4 hrs when compared to model control group. Treatment with diclofenac and saponin rich fraction of *S. xanthocarpum* showed significant reduction in paw volume at administered doses from 1 hr to 4 hr.

3.9.2. Evaluation of diuretic activity of saponin rich fraction

The supersaturation of urine with CaOX, the most common component of kidney stones, is an important factor in crystallization, with later factors being nucleation, growth and aggregation. Thus if supersaturation or later steps in crystallization can be prevented, then lithiasis should be avoided.[42] Urinanry volume was significantly increased in treatment groups i.e. frusemide, saponin rich fraction of *P. murex* and *S. xanthocarpum* as compared to control group. Treatment with frusemide significantly increased the excretion of sodium and potassium ion in urine compared to control while excretion of sodium significant at 80 mg/kg of *P. murex* and 20 mg/kg and 40 mg/kg of *S. xanthocarpum*

treatment. Potassium ion was significantly excreted at higher dose of *P. murex* and *S. xanthocarpum* but not at lower dose.

3.9.3. Evaluation of *in vitro* spasmolytic activity of saponin rich fraction

Spasmolytic activity is very important for passing and/or expelling the stone from renal system. *In vitro* spasmolytic activity of saponin rich fraction was done using acetylcholine and $BaCl_2$ as agonist. Dose response curves were recorded of both these agonist in the presence of varying concentration of saponin rich fraction of *P. murex* and *S. xanthocarpum* and found out % inhibition. Saponin rich fraction of *S. xanthocarpum* has higher inhibiting capacity as compared to *P. murex* against both acetylcholine and $BaCl_2$.

3.9.4. Evaluation of *in vitro* antioxidant activity

The role of active oxygen and free radicals in tissue damage in such diseases, are becoming increasingly recognized of which cancer, cirrhosis, arteriosclerosis, arthritis, renal disorder have all been correlated with oxidative damage. Active oxy- gen, either in the form of superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), or singled oxygen ($^1O_2$), is a product of normal metabolism and attacks biological molecules, leading to cell or tissue injury. Therefore, *in vitro* antioxidant activity of saponin rich fraction of *P. murex* and *S. xanthocarpum* was performed. From the result it was found that saponin rich fraction of both plants produce good *in vitro* antioxidant activity.

3.9.4.1. Effect of saponin rich fraction on DPPH radical scavenging activity

The antioxidant properties of saponin rich fraction of *P. murex* and *S. xanthocarpum* led to decreases the concentration of DPPH radical. The IC_{50} values were found to be 42.78, 134.23 and 87.68 µg/ml for ascorbic acid, saponin rich fraction of *P. murex* and *S. xanthocarpum* respectively.

3.9.4.2. Effect of saponin rich fraction on H_2O_2 radical scavenging activity

With H_2O_2 radical, ascorbic acid and saponin rich fraction of *P.* murex and *S. xanthocarpum* exhibited 50% scavenging activity at 64.59, 230.43 and 138.89 µg/ml, respectively.

3.9.4.3. Effect of saponin rich fraction on metal chelating activity

The metal chelating effect on the ferrous ions by the saponin rich fraction of *P. murex* and *S. xanthocarpum* showed as 0.82 and 1.3 mg of EDTA/ g of extract respectively.

3.9.4.4. Effect of saponin rich fraction on reducing power

The reducing capacity of saponin rich fraction of *P. murex* and *S. xanthocarpum* was increased with increasing concentration, though it was less than that of ascorbic acid.

4. CUNCLUSION

Out of four different extracts prepared from fruits of *P. murex* using petroleum ether, ethyl acetate, methanol and water, methanolic extracts produced better preventive activity against experimentally induced urolithiasis *in vivo* by decreasing supersaturation due to diuresis, decreasing hyperoxaluria and hypercalciuria, increasing excretion of citrate and by producing antioxidant activity. In this study, methanolic extract of *P. murex* showed preventive activity with *in vitro* calcium oxalate crystallization by decreasing nucleation, growth and aggregation, which are important for the development of kidney stone.

Similarly, saponin rich fraction prepared from *P. murex* produced preventive activity against experimentally induced urolithiasis *in vitro* and *in vivo* as mentioned above as well as by producing anti-inflammatory and spasmolytic activity, later one may help the stone easily pass through the urinary system harmlessly.

Four different extracts prepared from fruits of *S. xanthocarpum* using petroleum ether, ethyl acetate, methanol and water, out which methanolic extracts produced better preventive activity against experimentally induced urolithiasis *in vivo* by decreasing supersaturation due to diuresis, decreasing hyperoxaluria and hypercalciuria, increasing excretion of citrate and magnesium and by producing antioxidant activity. In our study, methanolic extract of *S. xanthocarpum* showed preventive activity with *in vitro* calcium oxalate crystallization by decreasing nucleation, growth and aggregation, which are important for the development of kidney stone.

Saponin rich fraction prepared from *S. xanthocarpum* produced preventive activity against experimentally induced urolithiasis *in vitro* and *in vivo* as mentioned above as well as by producing anti-inflammatory and spasmolytic activity, later one may help the stone easily pass through the urinary system inoffensively.

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